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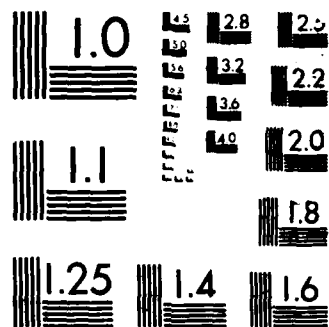
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WOUND COVERAGE BY CULTURED SKIN CELLS

ANNUAL SUMMARY REPORT

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ELLEN M. DUFFY, Ph.D.

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<p>The major purpose of our work is to refine the technology for growth of human epidermal cells to achieve more rapid growth <u>in vitro</u> and easier handling of tissue cultured materials in clinics. It is also to evaluate the possibilities for the use of allogeneic epidermal cells grown <u>in vitro</u> for wound healing.</p> <p>For the studies <u>in vitro</u> we used various tissue culture techniques. Quantitative data were obtained by cell counts and incorporation of radioactively labeled precursors, and qualitative data by light and electron microscopy. Studies <u>in vivo</u> were performed on experimental animals - swine. Careful visual observations were documented (continued over)</p>					
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histologically and by photography. They were supplemented by immunological approaches such as immuno-rosetting and studies of lymphocytes (MLC).

We have defined conditions for growth of epidermal cells on collagen matrices. Similarly, we have optimized conditions for preparing "dermal" grafts which are composed of collagen sponges (collagen type I) and fibroblasts. It was shown that such materials are suitable for transplantation and that their handling in clinical settings is acceptable. We have also shown that healing by epidermal cells grown on collagen is equal, if not superior, to the use of epidermal cells without a collagen substrate. Moreover, the combination of "dermal" grafts and epidermal grafts resulted in healing of some deep, chronic wounds.

Search for factors that would stimulate the growth rate of epidermal cells in tissue culture resulted in findings, that WI-38 cell extracts and an astrocytoma cell extract had pronounced growth stimulating effects. Further investigations of the growth promoting effect are needed prior to its inclusion into a protocol for growth of normal keratinocytes.

For the studies of allogeneic grafts, major emphasis was placed on development of techniques for rapid detection of major histocompatibility antigens (SLA) and identification of skin specific antigens. Immuno-rosetting with protein A coated erythrocytes proved a sensitive and specific method. Skin specific antigens were revealed on keratinocytes using sera obtained by skin transplantation between SLA matched animals. The antigens were not detectable on lymphocytes, fibroblasts and melanocytes. Their expression was shown to vary during their growth in vitro. Our transplantation experiments suggest a possible role for the skin specific antigens in skin rejection.

SUMMARY

The major purpose of our work is to refine the technology for growth of human epidermal cells to achieve more rapid growth in vitro and easier handling of tissue cultured materials in clinics. It is also to evaluate the possibilities for the use of allogeneic epidermal cells grown in vitro for wound healing.

For the studies in vitro we used various tissue culture techniques. Quantitative data were obtained by cell counts and incorporation of radioactively labeled precursors, and qualitative data by light and electron microscopy. Studies in vivo were performed on experimental animals - swine. Careful visual observations were documented histologically and by photography. They were supplemented by immunological approaches, such as immuno-rosetting and studies of lymphocytes (MLC).

We have defined conditions for growth of epidermal cells on collagen matrices. Similarly we have optimized conditions for preparing "dermal" grafts which are composed of collagen sponges (collagen type I) and fibroblasts. It was shown that such materials are suitable for transplantation and that their handling in clinical settings is acceptable. We have also shown that healing by epidermal cells grown on collagen is equal, if not superior, to the use of epidermal cells without a collagen substrate. Moreover, the combination of "dermal" grafts and epidermal grafts resulted in healing of some deep, chronic wounds.

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FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

Studies with human subjects are not a part of this contract. Results in this report obtained in collaboration with other investigators are only for illustration and are quoted with the approval of the collaborators.



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A. GROWTH OF EPIDERMAL CELLS ON COLLAGEN SPONGES

Sheets of epidermal cells used for transplantation can be:

1) peeled from the plastics, or 2) detached from the surface using enzymes such as dispase. Both procedures require much time and highly trained personnel, to prepare the grafts just prior to transplantation to the patients. In an attempt to simplify this procedure, we wanted to grow epidermal cells on collagen sponges. In a collaborative approach with F. Silver of Rutgers University, we have coated plastic Petri dishes with type I, III and IV collagens and evaluated epidermal cell attachment and growth. These preliminary experiments showed all above collagens to be suitable for growth of epidermal cells, and led to experiments using collagen sponges as a substrate.

When epidermal cells were seeded on collagen sponges having interstices of a pore size from 3 to 100 μ m, we have found that epidermal cells were lost in interstices and a confluent layer was not obtained.

We have therefore attempted to coat such collagen sponges with collagen Type III and IV, assuming that the material would provide a more suitable environment and fill the interstices. In a series of experiments, in which we used different amounts of coating material we have shown that even with coating of collagen sponges with Type III or IV collagen, epidermal cells would not properly attach and spread on the top of the collagen sponges. We have then attempted to coat collagen sponges with the plasma of the recipient. This coating improved the plating of epidermal cells, however, not sufficiently.

In a different series of experiments collagen sponges, both uncoated and coated with type III and IV collagen were collapsed and subsequently used to grow epidermal cells. Labeling experiments, measuring 3 -thymidine uptake have revealed that uncoated collapsed collagen sponges were equal to coated collapsed sponges in their support for growth of epidermal cells. Since coating of collagen sponges adds to the cost of preparation of such material and they do not offer growth advantage for epidermal cells, we have used uncoated collapsed type I collagen sponges to grow epidermal cells.

B. GROWTH OF FIBROBLASTS IN COLLAGEN SPONGES

To assess the possibility of providing dermal elements for treatment of deep wounds (corresponding to third degree of burn) we have studied growth of fibroblasts in collagen sponges and their transplantability.

Growth of fibroblasts in tissue culture.

To obtain sufficient numbers of fibroblasts for seeding the collagen sponges, the cells were obtained by growing them in vitro. A small (3 mm) punch biopsy from any area of the body cleaned with 70% alcohol, was delivered to the laboratory in Eagle's minimum essential medium (EMEM). If the sample was delivered immediately, the medium did not contain antibiotics or fetal calf serum. However, if storage for 4-6 hours was required, the medium contained antibiotics (penicillin 100 units/ml, streptomycin 0.1 mg/ml and fungizone 0.25 ug/ml) and fetal calf serum (5%) and the sample was kept at 4°C.

In the laboratory, the tissue specimen was washed in EMEM containing 10X more antibiotics than the transfer medium. The sample was then cut into multiple small pieces and attached to the bottom of a tissue culture Petri dish. The attached pieces were overlayed with EMEM containing 10% fetal calf serum and incubated in water-saturated-air containing 5% CO₂ at 37°C. Outgrowth of fibroblasts from the tissue was achieved in approximately 7-10 days. The cells were then detached from tissue culture dishes using trypsin and seeded into tissue culture flasks for further expansion. At this point the cells were replicating logarithmically, with cell cycle time of approximately 24 hours. Human and pig skin fibroblasts, used in our studies, have a limited life span. In general, we have found, that most cultures could be carried through approximately 15-20 passages. At each passage the cells were split in 1:3 ratio. In practical terms it means that from one 3 mm punch biopsy we could obtain in 15 passages 4 million flasks of cells with 18×10^6 cells/flask.

Seeding of collagen sponges by fibroblasts.

In previous experiments we have shown that fibroblasts can be seeded into knitted Dacron velour prosthesis. Growth of fibroblasts in these three dimensional matrices was very satisfactory. The cells, their attachment and growth could be monitored by inverted phase contrast microscopy. By trypsinization and cell counts we could show that the cells in the matrix

were replicating until they reached two dimensional contact inhibition. Such seeded matrices were used for reconstruction of anterior cruciate ligaments in dogs. The above mentioned studies were used as a model in the studies of fibroblast growth in collagen matrices. To shorten the incubation time required for ingrowth of fibroblasts into a three dimensional collagen matrix and to avoid partial degradation of collagen by the collagenase produced by fibroblasts we have chosen high seeding density of fibroblasts for the collagen sponges. A collagen sponge (2 mm thick) 80 mm in diameter was seeded with $3-4 \times 10^6$ fibroblasts. (Number of cells is 6-8 X higher than used for seeding a plastic dish of the same size). The cells in collagen can not be counted by conventional tissue culture methods. Therefore to evaluate the growth properties of the cells we have used ^3H -thymidine incorporation. Variation in the behavior of cells from different individuals were noted. However, in general peak DNA synthesis was seen 3-4 days after initial plating of cells. On the third day after seeding the cells, ascorbic acid (1 mg/ml) was added, since it was shown previously, that ascorbic acid will stimulate fibroblasts to produce collagen. Preliminary experiments suggests that ascorbic acid did not influence DNA synthesis in cells grown in collagen matrices, but slightly reduced DNA synthesis in cells grown on tissue culture plastics.

C. TRANSPLANTATION OF CELLS GROWN ON COLLAGEN MATRICES

Fibroblasts

Fibroblasts grown within collagen matrices were used for autologous transplantation on to a full thickness wound. Since our experimental animal model did not provide a chronic non-healing wound, we have chosen to use them for patients with chronic deep leg ulcers (in collaboration with M. Carter, M.D. at the Rockefeller Institute) for the evaluation of the wound healing effect of this material. Ten patients with leg ulcers who failed to respond to any conventional treatment were chosen for this study. The underlying cause of the ulcers varied. In all instances, we have found that the fibroblasts-seeded collagen grafts took and the deep wound was filled with granulation tissue. In three out of ten patients one or two applications of the fibroblasts-ingrown collagen sponges, in two patients followed by one application of an autologous epidermal graft, resulted in a permanent wound closure. In one patient, the application of the sponge with fibroblasts alone was a sufficient

stimulus to fill the wound and allow the epidermal cells to slide over the filled area and cover it.

Epidermal cells

As mentioned previously, the major aim of growing epidermal cells on collagen substrates was to facilitate the preparation of grafts for transplantation. Moreover, we thought that the period of time required to obtain multilayered sheets of cells which can be removed from tissue culture flasks and used for transplantation can be shortened. We have, therefore, seeded the collagen sponges with high cell density and used them for transplantation 5 days or 10 days after original seeding. The collagens used for these studies were not the collapsed ones. We have found that epidermal cells were trapped into the collagen and they did not form a nice layer on the top.

Based on more recent findings, that the collapsed collagen sponges provided the best base for the growth of epidermal cells, we have grown epidermal cells on them to confluency and then used them for transplantation. We have also found, that such collagens had to be very thin (approx. 1 mm thick), in order to be successfully transplanted and the cells to be properly nourished. Collagens which had multilayered epidermal cells growing on top (after 2-3 weeks in tissue culture) were successfully transplanted in pigs, also on a burn patient (in a collaborative work with Dr. Hartford in Chester, PA) and on two patients with epidermolysis bullosa (in collaboration with Dr. M. Carter, Rockefeller University, New York). The use of the epidermal cells grown on collagen for patients with epidermolysis bullosa was very successful. One patient's forehead, cheeks and ears were successfully covered and full epithelial coverage was achieved. The second patient, whose chin was covered recently, has colonies of epidermal cells growing on the originally denuded area. We are currently further improving this system and plan to establish the minimum time requirements for growth of epidermal cells on collapsed collagen sponges prior to transplantation.

D. IN VITRO TESTS OF GROWTH STIMULATING FACTORS FOR EPIDERMAL CELLS

We have recently shown that human melanocytes can be stimulated to grow in vitro by growth factors present in the cell lysates of normal fibroblast, malignant melanoma or astrocytoma cells. (1) Such factors are absent or present in nondetectable amounts in tissue culture supernatant

fluids.

Cell lysates of 3T3 and Wi-38 cells, astrocytoma cell lines and of normal keratinocytes were tested for growth stimulation of human keratinocytes. The rationale for choosing these particular cell lysates was as follows: 3T3 cells (mouse embryonic fibroblasts) have been shown previously (2) to be the most suitable feeder layer cells for the growth of human epidermal cells. Wi-38 cells (human embryonic lung fibroblasts) were chosen as a different fibroblast cell line of human origin, since it is generally suspected that fibroblasts (as dermal components) might play an important role in the growth of epidermal cells. Astrocytoma cell lines were used since we have previously found that they stimulate the growth of epidermal cells. Epidermal cell lysates were used since we have previously shown that successful growth of epidermal cells was dependent on the seeding density, and underseeded cultures resulted in an abortive growth. The growth stimulating effect of these cell lysates was compared to the effect of the epidermal growth factor (EGF) and insulin. As an assay system we have used ^3H -thymidine incorporation by the epidermal cells and cell counts. Our experiments suggest the strongest growth potentiating effect from the glioma and Wi-38 cell extracts. Surprisingly, 3T3 cells had very little effect on the growth stimulation of epidermal cells in the culture conditions generally used by us (3). Further experiments are now in progress to define the strongest mitogens for epidermal cells and their potential use for growth stimulation in vitro and in vivo.

E. ALLOGENEIC SKIN TRANSPLANTATION

Skin Specific Antigens

Previous studies have shown that skin allografts are rejected between donor and recipient combinations which allow for successful transplantation of other organs. Swine, inbred for the major histocompatibility complex, SLA, have been shown to reject skin drafts while maintaining a previously accepted kidney allograft from the same donor. (4) Expression of tissue restricted antigens on keratinocytes, skin specific antigens, has been proposed as an explanation of this rejection. The aim of our studies was to find whether epidermal cells express antigens not present on other cell types and whether growth in tissue culture causes changes in their expression.

The skin specific sera tested in this series of experiments were

obtained from the laboratory of David Sachs at NIH. They were derived as part of a study there in which swine of an inbred herd that frequently accept kidney allografts from SLA matched donors, without the requirement for immunosuppression, were tested for the effects of pre-sensitization by skin transplants from the future kidney donor. Sera were obtained pre-immunization and fourteen days after each skin or kidney transplant. There were three series of immunizations, two between donor/recipient combinations which were of identical SLA type dd and one between swine of SLA type cd.

Cells to be tested for expression of SLA and skin specific antigens were plated at a concentration of 5×10^5 cells/ml on Concanavalin-A coated wells of Terasaki plates. The relevant sera were tested by an immuno-rosetting assay using protein A coated human red blood cells as indicators.

The epidermal cells used for testing were prepared by trypsin splitting of the epidermis from the dermis and preparation of a single cell suspension. The cells were then incubated for 24 hours prior to testing to eliminate the effect of the enzyme on antigen expression. Lymphocytes were obtained as a mononuclear fraction from Ficoll Hypaque. Fibroblasts and melanocytes tested were grown by methods generally used in our laboratory.

Antigens present on epidermal cells, but not detectable on lymphocytes, fibroblasts, or melanocytes have been found by the protein A rosetting technique using sera described above. These antigens, which fulfill the definition of skin specific antigens, have been detected on the epidermal cells of twelve swine out of nineteen swine tested. Eleven of the swine which were positive for these antigens were from the same NIH-developed inbred herd as the swine used for the immunizations. One swine which expressed a skin specific antigens was an outbred Yorkshire from Quaker Farms, Quakertown, Pennsylvania. The cells of one outbred swine were negative for the skin specific antigens, although reactive with SLA typing sera. The lymphocytes of all swine tested were negative for the skin specific antigens, although the appropriate SLA were detected.

Within each immunization series, the pre-transplant serum and the first post-skin transplant serum did not detect the antigens. Significant antibody titers were found in sera collected two weeks after the

second skin transplants, with some decrease in titer at later times observed in the post-kidney transplant sera. The sera with the highest titer to antigens detected on keratocytes were chosen as representative sera for each immunization series.

As shown in Table I, representative sera of immunization series I, II and III detected different antigens. The pattern of reactivity of individual swine with the sera also suggests the possibility, that a single genetic locus for skin specific antigens, with alleles denoted here as p, q, r and u (for those presumably not detectable with the sera available). Among the swine tested there were two sets of SLA identical siblings: swine 1617, 1618, 1620, 1621 and swine 1412 and 1413. In both cases the siblings expressed different skin specific antigens.

Table I: Skin Specific Antigens

Epidermal cells (Pig No.)	Immunization Series			alleles
	I	II	III	
1567	+			p,u or p,p
1620		+		u,q or q,q
8		+		u,q or q,q
1413	+	+		p,q
1582	+		+	p,r
1412	+		+	p,r
1414	+		+	p,r
1110	+		+	p,r
1583		+	+	q,r
1618		+	+	q,r
1414		+	+	q,r
1617	+	(+)*	+	p,r

*Pig 1617 cells were reactive with the pre-immune as well as post-immune sera from the immunized animal of series II.

Expression of Skin Specific Antigens in Tissue Cultured Cells.

Keratinocytes of pigs 1412, 1413, and 1414 were tested in the protein A rosetting assay at various times after maintenance in tissue culture. At early times in culture, up to 14 days, the titers of reactivity with skin specific sera were comparable with those of fresh

epidermal cells. Cells cultures more than thirty days showed changes in the expression of skin specific antigens. The antigen(s) detected by series I immunization did not show significant modulation. However, the antigen(s) of series II immunization expressed by swine 1413 and those of series III expressed by swine 1412 and 1414 were not detectable. It is of interest, that the expression of major histocompatibility antigens detected using the same above assay system, did not change during the cultivation.

Allogeneic Skin Transplantation Results.

We have shown repeatedly, that epidermal cells grown in tissue culture when transplanted to an allogeneic host will be rejected, but that the rejection is delayed 7-9 days. We have also shown, that if epidermal cells are grown in the presence of a dermal extract of the recipient there is not visible rejection. Therefore our allogeneic pig skin transplantation studies were done with cells grown in the presence of dermal extracts.

In one series of experiments two pigs of the SLA type ad, (Pig 1413 and 1412) received transplants of prospective recipient dermal extract treated tissue culture epidermis from the same donor of SLA type ac, (Pig 1414). By visual examination and histology both recipients appeared to accept these transplants, although they differed in their matching of skin specific antigens with the donor. (Fresh epidermal donor cells (Pig 1414) expressed antigen I, III; Pig 1413 expressed antigen I, II, and Pig 1412 expressed antigens I, III). As mentioned previously, antigen II, which Pig 1413 did not share with Pig 1414 was lost during cultivation. On subsequent challenge with transplants of fresh epidermal cells (3 months after the first transplant) from the same donor (Pig 1414) the recipient Pig 1412 with skin antigens matching those of the donor, accepted two subsequent fresh epidermal transplants. In contrast, the recipient 1413, lacking one of the skin specific antigens rejected this graft within 4 days and also showed rejection reactions at the sites of the previously accepted tissue cultured grafts.

These transplant experiments seem to provide evidence for a role of skin specific antigens in the differential responses to tissue cultured, versus fresh epidermal grafts. It is now necessary to further document these preliminary observations.

In another series of experiments we have attempted to change the concentration of the allogeneic dermal extract used to grow the epidermal cells. Three pigs which were transplanted with such cells all rejected their grafts 14-16 days post-transplantation. The autologous grafts done at the same time were however accepted. We therefore plan to do titration experiments to establish the minimum amount and minimum time needed to "condition" the epidermal cells.

Effects of Epidermal Grafts on Mixed Lymphocyte Reactions.

Mixed lymphocyte cultures between swine matched for the major histocompatibility complex, SLA, or between swine of different SLA types, were carried out pre- and post-skin transplantation. The incorporation of ^3H -thymidine by pre-transplant cells cultured with autologous pre-transplant stimulating cells was used as a control for effects due to skin transplants. Two recipients, Pigs 1412 and 1413, both with SLA type ad, accepted dermal extract-treated tissue cultured epidermal grafts from the same donor, Pig 1414.

Recipient 1, Pig 1412, which by visual examination and by histology accepted the skin transplants, exhibited a higher response to the donor lymphocytes in the in vitro assay than did Recipient 2, Pig 1413, both pre- and post-transplantation. The subpopulation of lymphocytes which is proliferating as a result of the transplants is not known, and in this case may include suppressor cells.

Recipient 2 accepted the tissue culture graft but rejected the fresh epidermal cells. This appears to be reflected in the relatively low increase in proliferative response to donor lymphocytes following the tissue culture transplant and the significant rise following the fresh epidermal graft.

Perhaps of more interest is the increase in autologous response of Pig 1413 following the transplants. In contrast, the autologous response of Pig 1412 increased only slightly. It is proposed that the increase in proliferative response is due to an activation of the lymphocytes as a result of the rejection process causing them to be more effective as stimulators. The SLA identical Pig 1412 also showed an increased response to Pig 1413 lymphocytes. The correlation of increased autologous mixed lymphocyte response with rejection of skin grafts is being investigated in additional transplant experiments.

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